FTD-ID(RS)T-1322-89

FOREIGN TECHNOLOGY DIVISION

AIR FORCE SYSTEMS COMMAND

CHINESE JOURNAL OF BIOTECHNOLOGY

FOREIGN TECHNOLOGY DIVISION

Approved for public release;
Distribution unlimited.

HUMAN TRANSLATION

FTD-ID(RS)T-1322-89 2 April 1990

MICROFICHE NR: FTD-90-C-000317

CHINESE JOURNAL OF BIOTECHNOLOGY

English pages: 22

Source: Sheng Wu Kung Chaeng Hsuueh Pao,
Vol. 5, Nr. 1, 1989, pp. 11-18; 19-23

Country of origin: China
Translated by: Leo Kanner Associates
F33657-88-D-2188
Requester: FTD/TTTRL/Lt Billiana Owens
Approved for public release; Distribution unlimited.

Accession For	
NTIS GRA&I	☑
DTIC TAB	☐
Unannounced	☐
Justification	
By	
Distribution/	
Availability Codes	
Dist	Avail and/or Special
A-1	

PREPARED BY:

TRANSLATION DIVISION
FOREIGN TECHNOLOGY DIVISION
WPAFB, OHIO

TABLE OF CONTENTS

CLONING AND EXPRESSION OF δ-ENDOTOXIN GENE OF *BACILLUS THURINGIENSIS* IN *E.COLI*

Tian Yingchuan, Cai Faxing: Institute of Microbiology, Academia Sinica, Beijing
Wang Ying, Zhang Binxian, Sha Cayun: Institute of Zoology, Academia Sinica, Beijing
Chen Shouyi, Mang Keqiang: Academia Sinica, Beijing

Received 16 December 1987.

ABSTRACT [transcribed verbatim from English original]

Fragments larger than 4 kb from Sau$_3$A$_1$ partial digested plasmid DNA of *Bacillus thuringiensis* subsp. *kenyae* 7404 as well as subsp. *kurstaki* HD-$_1$ were cloned respectively into the BamH I site of pBR$_{322}$. Based on the results of in situ colony hybridization, radioimmune screening and Western blot analysis, four transformants containing the corresponding δ-endotoxin gene and producing proteins reacted with crystal protein antibody were selected. Upon biological toxicity tests, out of three transformants tested, the lysate of one transformant TK89 carrying δ-endotoxin gene of *B.T.* 7407 and two transformants TH12 and TH48 carrying δ-endotoxin gene of HD-$_1$ were toxic to caterpillars of tobacco budworm (*Heliothis assulta*). This is the first time to have cloned the δ-endotoxin gene of *B.T.* subsp *kenyae* different in serotype with the well studied subsp. ~~*kurstaki.*~~ Key words: *Bacillus thuringiensis*; DNA cloning, δ-endotoxin, CHINA.

Translator's notes:
For bibliographic references, see page 14.
For explanation of plate [not included], see page 14.

Notes in original:
The crystal protein and the antisera were provided by Bai Cheng, Feng Xichang, and Wen Jie of the Academia Sinica Institute of Zoology. Zhang Shufang and Xie Zhangjiang participated in the toxicity assay. Yang Haozhou of the Academia Sinica Institute of Microbiology and Li Zhanggong of the Chinese University of Science and Technology participated in a portion of the work. We wish to thank them all at this time.

This study is a topic subsidized by the National Science Committee.

ABSTRACT [translated from Chinese original]

Plasmids of *Bacillus thuringiensis* subsp. *kenyae* 404 and *Bacillus thuringiensis* subsp. *kurstaki* HD-1 were isolated. Through in situ gel hybridization, we have shown that the δ-endotoxin gene of *B.t.* subsp. *kenyae* 7404 is located on a plasmid about 47 Md in size. Using the sucrose concentration gradient centrifugal method, we isolated DNA fragments larger than 4 kb from Sau_3A_1 partially digested plasmid DNA of the two *B.t* subspecies. We cloned these fragments on the Bam HI site of pBR_{322}, and transformed *E.coli* HB_{101}. Through methods like in situ colony hybridization, in situ colony radioimmune testing, and Western blot analysis, genes carrying δ-endotoxin were selected, as well as transformants that could express this toxic protein in *E.coli*. Initial biological tests show that among four tested transformants, the transformant TK_{89}, carrying the *kenyae* subsp. δ-endotoxin gene, and transformants TH_{12} and TH_{48}, carrying the *kurstaki* subsp. δ-endotoxin gene, had a toxic effect on the tobacco budworm (*Heliothis assulta*).

Bacillus thuringiensis is a positive Gelanshi [transliteration] bacterium that is able to form spores. While forming spores, it also produces a kind of semi-spore cyrstal protein, δ-endotoxin. A large portion of the semi-spore crystal protein of *B.thuringiensis* has a toxic effect. In order to understand better and make use of this crystal protein, in recent years, as the technology for genetic engineering developed, several laboratories have undertaken cloning of the δ-endotoxin of different subspecies of *B.thuringiensis* and have obtained expression of *B.thuringiensis* in *E.coli* bacteria [1-3].

The toxic proteins produced by different subspecies of *B.thuringiensis* show differences in their toxicity and range of insecticidal capability [4,5]. In comparison with the *B.thuringiensis* subspecies *kurstaki* HD-1 (hereafter called simply HD-1), which is internationally in the most widespread use and has been investigated the most clearly, the *kenyae* 7404 subspecies (hereafter called the 7404) that has been isolated in China belongs to a different blood serum group, H4a4c. Its range of insecticide capabilities and toxicity, in comparison with HD-1, are at times even higher [4]. This paper reports on the plasmid DNA isolated from HD-1 and 7404, and compares the distribution and

position of δ-endotoxin genes in two kinds of plasmas of different origins. The DNA fragment carrying the δ-endotoxin gene was isolated and cloned.

I. MATERIAL AND METHOD

A. Material

1. Bacteria strains: The *B.thuringiensis* subsp. *kurstaki* HD_{-1} and *kenyae* 7404 were provided by the Institute of Zoology of the Academia Sinica; The 7404 was collected and isolated by the Institute of Zoology.

2. Biochemical reagents: For the restricting endonuclease and the other modified nuclease, we chiefly used BRL products or products purchased from the Huamei Company. The staphylococcus protein A is a Sigma product. The $Na^{125}I$ and γ-^{32}p-ATP are Amersham products. The CsCl was purchased from the Huamei Company or is a recovered and purified product of our own laboratory.

3. The *B.thuringiensis* crystal protein and its antisera were supplied by the Institute for Zoology.

B. Methods

1. Extraction of plasmid DNA and in situ gel hybridization:

(a) Extraction of *B.thuringiensis* plasmid DNA: This was implemented with reference to the method of Kronstad et al. [6] for extracting large plasmids. The extraction of recombinant plasmids was undertaken by the alkali method [7].

(b) Preparation of δ-endotoxin gene probes: Using an Applied Biosystems DNA synthesizer we synthesized isolated nucleotides of 25 nucleotides situated on the *B.thuringiensis* subsp. *kurstaki* HD_{-1}-Dipel crystal protein gene EcoRI *F* fragment. After 16% polypropylene acyl amine-urea gel electrophoretic purification, we used T4 multiple nucleotide stimulating enzymes and γ-^{32}p-

ATP to undertake marking according to the method presented by Maniatis and others [7]. These marked isolated nucleotides are the probes used for DNA molecule hybridization.

(c) In situ gel hybridization. After electrophoresis of the *B.thuringiensis* plasmid DNA on 0.8% agarose gel, we undertook in situ gel hybridization according to the Kidd method [8]. The probes used for the hybridization were the above-mentioned ^{32}P marked isolated nucleotide fragments.

2. Isolating and cloning of the enzyme section: About 20 µg of *B.thuringiensis* plasmid DNA and 0.15 units of Sau_3A_1, according to the manufacturer's recommendations, were subject to partial digestion at a temperature of 37°C for 15 minutes. After the digestion, the specimen was centrifuged for 20 hours at 15°C and 68,000 x g at 5-40% sucrose concentration gradients; then DNA fragments collected at positions of different concentration were distributed. Each distribution of 10 µl was subjected to 1% agarose gel electrophoresis; the size of the DNA fragments was checked, and for the distributions that were gathered and merged that contained DNA fragments larger than 4 kb, the DNA was recovered by ethanol precipitation and dissolved in a TE buffer solution. After electrophoresis testing, it was estimated that the recovered DNA concentration was 0.2 µg/µl.

The pBR_{322} carrier Bam HI was digested and dephosphorized according to the method described in "Molecular Cloning" [7]. *E.coli* HB_{101} sensitive state cells were prepared according to the method of Alexander et al. [9]. About 0.6µg of DNA fragments recovered by sucrose concentration gradient centrifuging and 0.2 µg of pBR_{322}, with Bam HI digested and dephosphorized, under the effect of T4 DNA linking enzyme, were left overnight for linking reaction at 4°C. Regarding the *E.coli* HB_{101} that were transformed according to the method of Alexander et al. [9], those which were transformed were selected on an ammonia benzyl penicillin plate; on a tetracycline plate, transformants that may have had DNA inserted from an extraneous source were selected.

3. Hybridization of *B.thuringiensis*: We took the clones that we obtained in our transformation experiments that were sensitive to tetracycline and placed them on a ammonia benzyl penicillin LB plate. Using a nitrocellulose filter membrane, we covered the plate and incubated it overnight at 37°C. Then we processed the filter membrane according to the method of Southern [10]. Before initial hybridization, we placed the filter membrane in 3 x SSC at 65°C overnight. We undertook initial hybridization at 42°C for 5 hours (initial hybridization fluid: 6 x SSC, 0.05% scorched sodium phosphate, 1 x Denhardts solution, 0.5% SDS, 100 µg/ml denatured fish semen DNA). Hybridization was carried out for 20 hours at 42°C (the hybridization liquid was similar to the initial hybridization liquid, except that it did not contain SDS, and probe material was added to a concentration $1x10^6$ cpm/ml. After hybridization, there were two washings at 42°C in 6 x SSC and 0.05% scorched sodium phosphate and two washings at 42°C in 2 x SSC and 0.1% SDS; after drying at room temperature, we implemented autoradiography.

4. In situ colony radioimmune assay and Western blot analysis.

(a) Preparation of *B.thuringiensis* crystal protein antisera adsorbed using *E.coli* and its bacteriolytic fluid: 450 ml of pBR_{322}/HB_{101} cells incubated overnight and 0.3 ml of 7404 crystal protein antiserum mixed together were suspended in 100 ml of PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 4.6 mmol/L Na_2HPO_4, and 1.5 mmol/L KH_2PO_4) and left overnight at 4°C. After 8,000 rpm centrifuging, the supernatant was extracted. To another 450 ml batch of pBR_{322}/HB_{101} cells that was incubated overnight was added 50 ml of splitting fluid (0.05 mol/L Tris-HCl pH 7.5, 12.5 mg bacteriolytic enzyme); this was mixed evenly and left for 30 minutes at room temperature, put on ice for 1 hour, and then frozen and melted three times and centrifuged 20 minutes at 3,000 rpm; the supernatant was extracted. To 33 ml of each of these two kinds of supernatant was added 33 ml of PBS; after even mixing it was left at 4°C overnight. After 20,000 G centrifuging at 4°C for 1 hour, the supernatant was recovered, and kept ready for use at 4°C. The plasma dilute after adsorption processing was nearly 1,000 fold.

(b) In situ colony radioimmune assay: As for the in situ colony hybridization, the colony is reimprinted on a nitrocellulose membrane and incubated overnight at 37°C. Then it is subjected to slight improvement with reference to the method of Erlich et al. [11]. After that, bacteriolytic and immune reactions are undertaken. For the immune reaction, we placed the filter membrane with the colony in PBS containing 1% BSA and *B.thuringiensis* crystal protein antisera diluted to 1:2,000 (after pBR_{322}/*E.coli* HB_{101} adsorption) and vibrated it for 2 hours at room temperature, causing the colony to adsorb the antisera. After washing the filter membrane with PBS four times, we placed the filter membrane in PBS that contained 1% BSA and 10^{6}cpm/ml of protein-A that had been ^{125}I marked using the ammonia-amine T method [11], and vibrated it at room temperature for 2 hours. Finally, we washed it five times with PBS; we could then undertake autoradiography.

(c) Western blot analysis: We prepared the clone bacteria general protein sample in accordance with the method of Thorne et al. [12]. The SDS-polypropylene acyl amine gel electrophoresis of the protein was carried out according to the method of Laemmli [13], except that we used a 10% polypropylene acyl amine-SDS linking gel. After electrophoresis, with reference to the method of Towbin et al [14], we transferred the protein electrophoresis strip to the Z-probe membrane. The transfer buffer solution was 25 mmol/L Tris and 192 mmol/L glycine, with a pH of 8.3. After transfer, we placed the Z-probe membrane in PBS containing 10% BSA and left it overnight at 48°C. The combining reaction with antisera and ^{125}I-marked protein A was carried out according to the method described in the section on "In situ colony radioimmune assay."

5. Biological assay of cloned bacteria toxicity. As for the section on "in situ colony radioimmune assay," we used the freezing and thawing method to prepare the cloned bacteria and the pBR_{322}/*E.coli* bacteriolysate. Weighing out 30 mg of bacteriolysate, we added an appropriate amount of distilled water to dissolve it, and added it to man-made feed (0.5 x 0.5 x 0.1 cm^{3}). This we fed individually to 10 second-stage caterpillars of the tobacco budworm

(*Heliothis assulta*); or smeared it on the surface of 500 mg of corn leaves and fed it to 20 third-stage specimens of *Leuca separta*. We set up a control with an equivalent amount of pBR_{322}/HB_{101} bacteriolysate. After 72 hours of observation we recorded the growth and mortality conditions of the experimental insects. We repeated each set of experiments two times.

II. RESULTS AND DISCUSSION

A. Isolation of *B.thuringiensis* Plasmid and In Situ Gel Hybridization

B.thuringiensis has over 20 subspecies. The number of plasmids contained by different subspecies shows dissimilarities, but in general it is between 2 and 12. Their δ-endotoxin gene code is on one or several large plasmids [15]. We extracted the plasmid DNA from *B.thuringiensis* HD_{-1} and 7404 according to the method described by Kronstad [6] for extracting large plasmids. Plate I-A [translator's note: Plate was not included in the material provided, but comments on the plate can be found on page 14] shows the electrophoresis for these two kinds of plasmids. As shown in the figure, the electrophoresis of plasmid DNA extracted from HD_{-1} has at least ten relatively clear plasmid strips (shown by "2" in plate I-A; its size and number are comparable with the results obtained by Kronstad et al. [6]. "3" in plate I-A shows the electrophoresis of 7404; it has at least seven plasmid strips. The plasmid of 7404 and HD_{-1} are basically similar; the difference is only that at locations 4.9 Md and 30 Md HD_{-1} has two strips while 7404 only has one strip; at about 150 Md, HD_{-1} has one plasmid strip and 7404 has two plasmid strips. The results of in situ gel hybridization autoradiography are provided in plate I-D, showing that the large plasmids 47 Md and 150 Md have an obvious hybridization with the ^{32}p-marked synthetic probes ("2" in plate I-D). This approaches the results of Kronstad et al. [11, sic]: The results of Kronstad include the appearance at location 47 Md of two hybrid strips (approximately 44 Md and 47 Md), while the results of this paper only show one strip. This may be because the molecular weight of these two plasmids are similar and cannot be separated. In 7404 plasmid DNA there is one hybridized strip (47 Md, "3" on plate I-D): this shows that the δ-endotoxin gene of 7404 is very

possibly carried by the 47 Md plasmid. The DNA probe and pBR_{322} show no hybridization ("1" on plate I-D), and in the electrophoresis only the lightly-dyed strip HD_{-1} 150 Md large plasmid appears to feature visible hybridization ("2" in Plate I-D). This fact shows that the synthetic gene probe has an idiosyncratic nature. Plates I-A and I-D are the same gel; the difference in the size of the photographs is a result of different enlargement factors.

B. Isolation and Cloning of Enzyme Sections.

The δ-endotoxin of *B.thuringiensis* is generally about 4 kb [16, 17]. In order to obtain a complete toxic protein gene, we used Sau_3A_1 on 7404 and HD_{-1} plasmid DNA to undertake partial digestion. Then we undertook isolation on the partially digested fragments using the 5-40% sucrose gradient centrifugal method. Plate I-C shows the 1% agarose electrophoresis of scattered collected samples after centrifuging; the distribution from large to small of the enzyme sections is rather even, and it can be seen that the partial digestion reaction's conditions are suitable. Portions containing fragments larger than 4 kb were pooled and the DNA was recovered by ethanol precipitation (lane 15 in plate I-C).

The DNA samples larger than 4 kb recovered by sucrose gradient were mixed at 3:1 with 5' end dephosphorized pBR_{322} Bam HI digested fragments; linking was undertaken with T4 DNA linking enzymes, and then *E.coli* HB_{101} sensitive state cells were transformed. From about 5,000 Amp^r HD_{-1} δ-endotoxin gene clones, 1,750 clones sensitive to tetracycline were obtained; from about 6,000 Amp^r 7404 δ-endotoxin gene clones, 1,750 clones also sensitive to tetracycline were selected. By means of rapid plasmid sampling extraction and electrophoretic testing, it was discovered that the majority of clones sensitive to tetracycline carried plasmids into which had been inserted extraneous DNA larger than 4 kb.

C. In Situ Colony Hybridization.

We undertook in situ colony hybridization with ^{32}P-marked δ-endotoxin gene probes on clones sensitive to tetracycline. Plate I-E shows the results of in situ hybridization autoradiography for 7404 δ-endotoxin gene clones. Although the non-specific hybridization produced a relatively deep background (this may be because the initial washing of nitrocellulose membranes bearing colonies was insufficient, or because after hybridization they were not washed thoroughly), nevertheless the positive hybridization and the non-specific hybridization colonies featured obvious differences. the clones of HD-1 plasmid DNA fragments also had similar obvious in situ colony hybridization results (unpublished material). After the above hybridization, 138 positive hybridization clones were selected from 1,750 7404 clones sensitive to tetracycline; and 50 positive hybridization clones were selected from 1,750 HD-clones sensitive to tetracycline.

D. In Situ Colony Radioimmune Assay and Western Blot Analysis

In preparing the experiment, we discovered that the *B.thuringiensis* 7404 crystal protein's antisera were also able to generate immune reactions with *E.coli* HB_{101} or HB_{101} bacteriolysates with pBR332. This greatly affected the specific nature of the immune reaction. This kind of non-specific reaction may be a result of the presence of *E.coli* antibodies in the rabbits used to produce the antisera. The use of pBR_{332}/*E.coli* HB_{101} cells and their cell splitting supernatant in sequence with the 7404 crystal protein antisera mixed together to adsorb the *E.coli* antibodies in the antisera is thus able to eliminate this non-specific reaction (refer to Plate I-F and G).

Plate I-F shows the results of undertaking the in situ colony radioimmune assay on the above-described positive hybridization for 7404 crystal protein antisera and ^{125}I-marked protein A. From the 7404 δ-endotoxin gene clones of the in situ colony positive hybridization were selected 74 positive immune reaction clones; in the same way, 20 positive immune reaction clones were selected from the HD-1 clones. In the autoradiography, the difference in the

depth of the stains of the positive clones perhaps reflected the difference in the level of the δ-endotoxin gene reached in each clone. It may also be because of the difference in the number of cells in each colony. For this reason, it can only be regarded as a qualitative assay. It was also possible to undertake immune reactions for the 7404 crystal protein antibodies with HD-$_1$ endotoxin gene clone strains; this showed that the toxic proteins of these two kinds of bacteria feature a similar antigen determinant cluster.

Plate I-B is the electrophoresis of the positive immune reaction portion of cloned plasmid DNA. Analysis of the other positive immune cloned plasmid electrophoresis also produced similar results, showing that all positive immune clones have extraneous DNA insertions larger than 4 kb. Therefore, these clones may carry intact δ-endotoxin genes or at least the relevant portion of coded antigen genes.

In order to compare corresponding amounts and molecular weight of all immune positive clones expressing δ-endotoxin proteins, we used the cell splitting material of each clone to undertake SDS-polypropylene acyl amine gel electrophoresis and Western blot analysis. After electrophoresis, we used kaomasi [transliteration] bright blue dye; it was possible to see in the electrophoresis that several clones had a weak toxic protein dye strip. This is explained by the fact that δ-endotoxin genes are expressed rather weakly in *E.coli*. We transferred the protein strip in the gel by electrophoresis to a 2-probe membrane, and undertook radioimmune assays. The results showed that there were four clones that produced very deep autoradiographs in the in situ radioimmune assay that likewise were able to form specific immune reaction strips in the Western blot. Their molecular weight was the same as for 7404 toxic protein (130 k). These four clones were HD-$_1$ toxic protein gene clones TH_{12} and TH_{48} and 7404 toxic protein gene clones TK_{89} and TK_{102}. Plate I-G is the Western blot autoradiography including these four clones. It can be seen that the toxic protein of one 7404 clone, TK_{118}, expression amount is too small to be detected on the autoradiograph; the TK_{88} and TK_{107} expression amounts were small; and the TK_{89}, TK_{102}, TH_{12} and TH_{48} all had obvious toxic protein strips. The TH_{48} toxic protein also had a certain splitting,

producing about 65 kd of polypeptide. The δ-endotoxin proteins expressed by these clones were all in the range of about 130 kd, the same as for the toxic protein molecular weight of 7404 and HD_{-1}. Because the samples used for electrophoresis were the supernatant of the cloned bacterial cells' splitting product, although the volume used was the same in all cases (6 μl), nevertheless we did not measure the density of the protein, so only a relative comparison is possible.

E. Biological Assay of Cloned Bacteria Toxicity.

The results of assays of toxicity on *Heliothis assulta* and *Leuca separta* (Table 1, Fig. 1) show that the TH_{12} and TH_{48} cloned bacteria toxic protein genes are expressed rather well, and had a strong toxic effect on the experimental insects; the expression of TK_{80} was not so strong as TH_{12} and TH_{48}, but still killed a small percentage of the insects, and clearly produced the phenomenon of growth inhibition. The body weight of the surviving TK_{80} *L.separta* specimens was 1/3 the weight of the pBR_{322}/HB_{101} control group. The TK_{80} group also had a clear growth-inhibiting effect on *H.assulta* (data not yet measured). Although the number of specimens was quite large, the food intake of the experimental group of larva was very small before the occurrence of the toxic reaction, after which food intake ceased. Initial estimates are that the toxicity of the toxic protein produced by cloned bacteria is comparable to the toxicity of the original *B.thuringiensis* crystal protein.

Table 1. Toxicity assay of clones.

[illegible]	TH_{12}	TH_{48}	TK_{80}	pBR_{322}/HB_{101} (对照)
[illegible] *H. assulta*	++++	++++	+	−
[illegible] *L. separata*	++++	++++	+	−

Note: ++++ means strongly toxic to insects tested; - means non-toxic to insects tested. Key: (1) Control.

The results described above are explained by the fact that the HD_{-1} and 7404 toxic protein gene has been cloned and is able to express protein with biological activity.

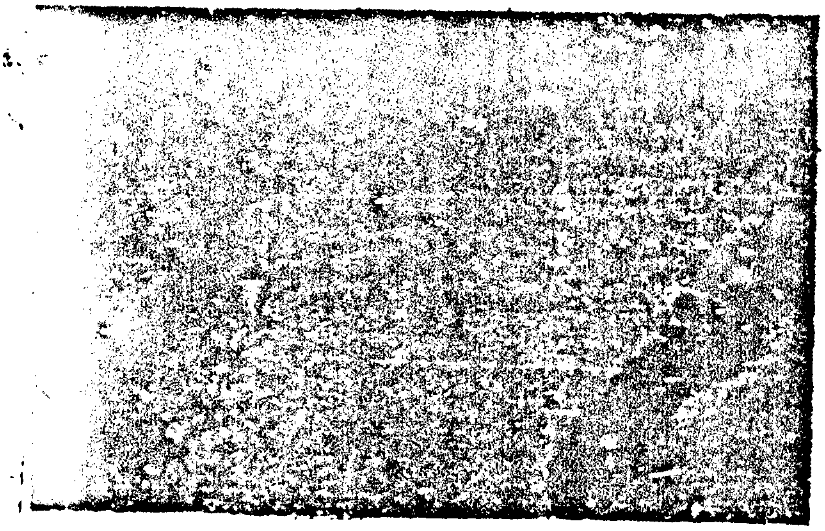

Fig. 1. Toxicity assay of clone TK_{a9} carrying δ-endotoxin gene of *B.thuringiensis* 7404. A. Larvae of *H.assulta* fed with diet containing lysate preparation of pBR322/*E.coli* HB_{101}; B. Larvae of *H.assulta* fed with diet containing lysate of preparation of clone TK_{a9}. (Pictures were taken after 48 hours feeding on the diet).

REFERENCES

1. Schnepf, H.E. and Whiteley. Proc. Natl. Acad. Sci., U.S.A., 78:2983, 1981.

2. Klier, A., et al. The EMBO Journal, 1, 791, 1982.

3. Honigman, A., et al. Gene, 42:69, 1986.

4. *Bacillus thuringiensis Research Group,* Institute of Zoology, Academia Sinica. Weishengwu Xuebao, 18:352, 1978.

5. Krieg, A., and G.A. Langenbruch. In Microbial control of pest and plant diseases 1970-1980 (H.D. Burges, ed.), pp. 837-896, Academic Press, London.

6. Kronstad, J.W. et al. J. Bacteriology, 154:419, 1983.

7. Maniatis, T. et. al. In Molecular cloning, a laboratory manual, CSH.

8. Kidd, V.J. Focus, 6:3, 1984.

9. Alexander, D.C. et al. Gene, 31:79, 1984.

10. Southern, E.M. J. Mol. Biol., 98:503, 1975.

11. Erlich, H.A. et al. Cell, 13:681, 1987.

12. Thorne, L. et al. J. Bacteriology, 166:801, 1986.

13. Laemmli, U.K. Nature, 227:680, 1970.

14. Towbin, H. et al. Proc. Natl. Acad. Sci. U.S.A., 76:4350, 1978.

15. Carlton, B.C. and J.M. Gonzales, Jr. In Molecular biology of microbial differentiation, J.A. Hoch and P. Setlow, edd., Washington, D.C., 1985, pp. 246-251.

16. Schnepf, H.E. et al. J. Biol. Chem., 260:6264, 1985.

17. Wabiko, H. et al. DNA, 5:305, 1988.

EXPLANATION OF PLATE

A. Gel electrophoresis of CsCl gradient purified *Bacillus thuringiensis* plasmid DNA on 0.8% agarose gel.

Lane 1. pBR322.
Lane 2. Plasmids from *B.T.* subsp. *kurstaki* HD-1.
Lane 3. Plasmids from *B.T.* subsp. *kenyae* 7404.

The numbers on the left indicate the sizes in Mdal of *B.T.* HD_{-1} plasmids. The white dots on the right of lane 2 and 3 indicate the plasmids hybridized with probe in D.

B. Gel electrophoresis of some recombinant plasmids from colonies with positive reaction in radioimmune screening. "TK" designates the clones of δ-endotoxin gene of *B.T.* subsp. *kenyae* 7404. "TH" designates the clones of δ-endotoxin gene of *B.T.* subsp. *kurstaki* HD_{-1}.

Lanes 1-5: Clones TK_{84}, TK_{162}, TK_{76}, TK_{63}, TK_{37}.
Lanes 6-12: Clones TH_{30}, TH_{48}, TH_{44}, TH_{37}, TH_{33}, TH_{28} and TH_{26}.
Lanes 13 and 14: $pPSST_{3425}$, a plasmid of 10.5 Kb in size and pBR_{322} respectively used as MW markers.
Lanes 15-18: Clones TH_{18}, TH_{15}, TH_{13}, TH_{12}.
Lanes 19-23: Clones TH_{2}, TK_{98}, TK_{118}, TH_{8}, TK_{89}.

C. Gel electrophoresis of DNA fragments of Sau_3A_1 partial digested *B.T.* subsp. *kenyae* 7404 plasmid DNA in different fractions of sucrose gradient.

Lane 1: λ-Hind III marker.
Lane 2: Plasmid DNA from *B.T.* subsp. 7404.
Lane 3: The same plasmid as in lane 2, but partially digested with Sau_3A_1.
Lanes 4-14: DNA fragments in different fractions of sucrose gradient.
Lane 15: DNA fragments from pooled fractions containing fragments larger than 4 kb.

D. In situ hybridization of agarose gel from A as described in Materials and methods. Samples in lane 1, 2 and 3 were the same as that in A. The numbers on the right indicate the size of plasmids hybridized with ^{32}p-labelled δ-endotoxin gene probe.

E. In situ colony hybridization: Colonies on nitrocellulose membrane were hybridized with ^{32}p-labelled probe and autoradiographed.

F. In situ colony radioimmuno-assay: Colonies hybridized with ^{32}p-probe in E were incubated with antisera against δ-endotoxin of *B.T.* 7404 and ^{125}I-protein A and autoradiographed.

G. Western blot analysis of proteins produced in δ-endotoxin gene clones.
1-9: Lysate of clone TH_{12}, TH_{48}, TK_{88}, TK_{118}. TK_{102}, TK_{107}, TK_{88}, pBR_{322}/HB_{101} and δ-endotoxin of *B.T.* 7404.
The numbers on the right indicate the sizes of protein molecular weight of δ-endotoxin.

CLONING AND EXPRESSION OF THE HEAT-LABILE ENTEROTOXIN GENE OF AN ENTEROTOXIGENIC *ESCHERCHIA COLI* HUMAN STRAIN

Chen Xingbo, Chen Tianmi, Institute of Biotechnology, Academy of Military Medical Sciences, Beijing
Ma Xiankai, Institute of Basic Medical Sciences, Academy of Military Medical Sciences, Beijing
Huang Cuifeng, Institute of Biotechnology

Received 10 September 1987.

ABSTRACT

DNA of the heat-labile enterotoxin (LT) plasmid pJY_{11} originating from H10407, an enterotoxigenic *Escherchia coli* human strain, was digested to completion with PstI. The location of the LT region on this plasmid was determined by means of Southern hybridization after electrophoretically separating the resulting fragments. The 5.3 kb LT-encoding fragment determined in the above hybridization experiment was then recovered and subsequently ligated to pUC_8 DNA predigested with PstI. After transformation and selection, a clone that efficiently expressed LT was obtained. Biological and immunological assays showed that LT produced by this clone was biologically and immunologically identical to that by the parental strain, and LT production level of this recombinant strain was 16 times higher. Key words: ETEC; heat-labile enterotoxin; cloning and expression.

Enterotoxigenic *Escherchia coli* (ETEC) is an important pathogenic bacterium causing infantile diarrhea and traveller's diarrhea. Its toxic factors are chiefly its adhesive element and its enterotoxic element. After ETEC has invaded the human body, it first established residence in the upper tracts of the small intestine, and then secretes enterotoxins, inducing diarrhea.

Translator's notes:

For bibliographic references, see page 22.

For explanations of the plate [not included], see page 23.

Notes in original:

Nan Li and others provided great assistance in the cytological experiments; Zhang Zhaoshan and others provided beneficial guidance in this work. At this time we wish to thank them all.

The heat-labile enterotoxin (LT) of ETEC is composed of one A subunit with an activation effect on glandular glucoside acid ring-forming enzymes and five B subunits with a combining effect [1]. It has been verified that the original immunological nature of ETEC pathogenic strains isolated in different regions is completely identical. The LT genes of human and swine ETEC also have a broad similarity in their original nature [2,3].

The Japanese scholar Yamamoto et al. were the first to report, in 1980, the cloning of the human ETEC LT gene; he also undertook studies of its structure [1,4]. But it was in China that the molecular genetics and epidemiology of ETEC were to be studied in depth. On the basis of the isolation and purification of the *E.coli* H10407 LT code plasmids, we achieved cloning and expression of its LT gene. The toxic gene clone obtained not only provides assistance in the survey of ETEC epidemiology, but also has laid a foundation for immune protection for traveler's diarrhea.

I. MATERIALS AND METHODS

A. Materials

1. Bacteria strains: *E.coli* JM_{83} (ara, Δlacpro, strA, thi, Φ80dlacZ M15) was the intestinal bacillus K_{12} receptor bacterium. *E.coli* 20SO (pJY_{11}) was the bacterium strain carrying the *E.coli* H10407 LT code plasmid, kindly provided by Dr. T. Yamamoto of the Shuntiantang [transliteration] University in Japan. *E.coli* H10407 is the internationally recognized standard strain of human ETEC. *E.coli* JM_{83} (pUC_{8}) is the carrier plasmid pUC_{8} carrier strain.

2. Bacteria culture medium: Common LB culture medium; toxin production culture base, prepared according to the literature [5]; and MacConkey lactose culture medium, a product of the Academy of Military Medical Sciences Laboratory Number 5.

3. Restricting endonuclease etc.: EcoRI, Hind III, PstI, XbaI I T4DNA ligation enzymes were products of the Huamei Biological Engineering Company. The low melting-point agarose IPTG was a product of the Sigma Company. The LT antisera were purchased from the Shanghai Sanitation and Epidemic Prevention Station. The ^{32}P-marked swine ETEC LT gene probe was supplied by Assistant Professor Yu Shouyi of the First Military Medicine University.

B. Methods

1. The preparation of the DNA and the construction and analysis of the recombinant plasmid: Extraction of the plasmid DNA was undertaken according to a modified Birnboim alkali transformation method [6]. For the digestion of restricting endonuclease and DNA external ligation and agarose gel electrophoresis, see the method described in reference [3]. Transformation of the DNA was carried out according to the method of Mandel and Higa (1970). Southern nucleic acid imprinting technology was carried out by the method of E. Southern [7]. For DNA fragment recovery, we used the low melting-point agarose method.

2. The LT semifinished product preparation and the passive immune hemolysis (PIH) test: Carried out per reference [8].

3. Testing of toxic biological activity: The domestic rabbit intestinal ligated loop assay and Chinese hamster ovary (CHO) cell assays were carried out according to references [9] and [10], respectively.

II. RESULTS AND DISCUSSION

A. Position Fixing of LT Gene

After completely digesting purified LT encoded large plasmid pJY_{11} with restricting enzyme PstI, we loaded it on 0.7% agarose gel. Using the Tris-boric acid buffer system with a voltage of 10 V/cm, we carried out electrophoresis for 3 hours to isolate the DNA fragments. Using 0.5 mol/L

NaOH, after transforming the DNA fragments on the agarose gel, we used the E. Southern method to transfer it to a nitrocellulose membrane. Then we hybridized it with a swine ETEC LT gene probe [11] (containing a portion of the A subunit position and all B subunit position code sequences). The results showed (Plate I-1) that the pJY_{11} third PstI strip was able to hybridize with the probe. Further, the experiment verified that these fragments were all about 5.3 kb. It showed that the LT gene was positioned on a 5.3 kb PstI fragment of the pJY_{11} plasmid.

B. Cloning of the LT Gene

Using PstI, we completely digested pJY_{11} plasmids carrying LT genes; then we loaded them on 0.7% low melting point agarose gel and undertook electrophoresis isolation. After dying with 0.5 µg/ml of ethidium bromide, we excised the third PstI strip under a long wave ultraviolet lamp. After melting the gel entirely at 65°C, we performed phenol extraction and alcohol precipitation. We mixed 0.3 µg of the recovered DNA with 0.5 µg of pUC_8DNA which had previously been completely digested with PstI, and performed ligation at 14°C with T4DNA ligation enzyme for 18 hours. The ligated compound was used for the transformation of sensitive-state *E.coli* JM_{83} cells. On a MacConkey lactose culture medium plate (containing 100 µg/ml ammonia benzyl penicillin; further, 10 µl of 0.1 mol/L IPTG solution was smeared on the plate surface), we selected 100 strains of anti-ammonia benzyl penicillin white colonies featuring extraneous DNA inserts. We randomly extracted 42 strains and undertook passive immunity hemolysis (PIH) testing. The results were that 23 strains were PIH test positive. The SPA coordinated agglutination results were identical with those of the PIH test. From the 23 PIH positive strains, we extracted 10 and undertook plasmid analysis, showing that the plasmids carried by all strains were larger than the carrier PUC_8. The explanation surely is that they have extraneous DNA insertions. The plasmid of purified recombinant *E.coli* JM_{83} ($pCHP_{31}$), after complete PstI enzyme dissection, bears out that it is possible to produce 5.3 kb and 2.7 kb fragments. After transformation of this DNA on agarose gel, we used the Southern method to transfer it to a nitrocellulose filter membrane; only 5.3

kb fragments were able to hybridize with the swine ETEC LT gene probe. The explanation is that $pCHP_{21}$ is surely a recombinant plasmid with the insertion of a 5.3 kb LT fragment. The other type of fragment produced by digestion is a carrier pUC_8; therefore it is not able to hybridize with the LT probe. In addition, we undertook EcoRI, PstI, and XbaI enzyme unitary and crisscross enzyme dissection analysis on $pCHP_{21}$, using the DNA fragments produced by λ DNA via EcoRI and Hind III digestion as the molecular weight standard. We measured the size of each fragment produced; results are shown on Plate I-1. The construction of $pCHP_{21}$ is shown in Fig. 1.

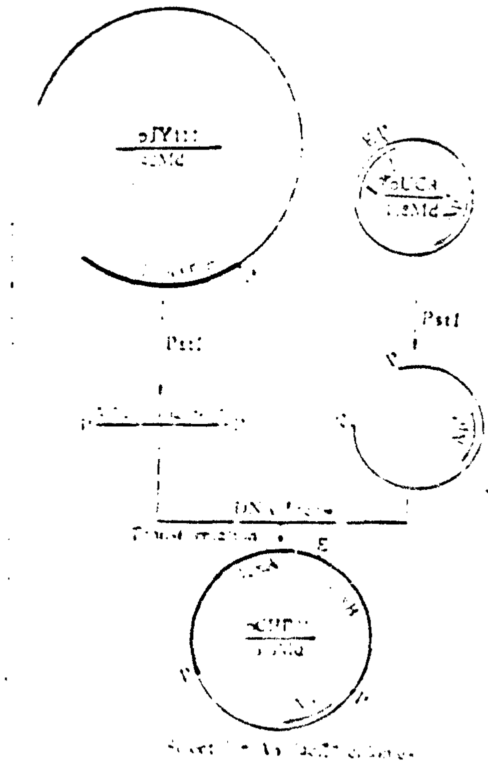

Fig. 1. Schematic diagram for the construction of $pCHP_{21}$. E: EcoRI; P: PstI; X: XbaI.

C. LT Gene Expression

The results of restriction analysis and Southern nucleic acid imprinting show that $pCHP_{21}$ is surely a recombinant plasmid carrying an LT gene fragment. But what are, in fact, are the conditions in which $pCHP_{21}$ expresses LT in the host bacterium? To answer this, we undertook semiquantitative measurement of the LT produced by the recombinant *E.coli* JM_{83} ($pCHP_{21}$). The results of the passive immune hemolysis experiment (Table 1) show that this cloned strain's LT production is 16 times as great as that of its parent strain, *E.coli* H10407.

Using the Chinese hamster ovary cell assay and the adult rabbit intestinal ligated loop assay, we undertook biological activity tests on the LT produced by the LT clone strain. Activated LT causes the Chinese hamster ovary cells to undergo morphological changes, showing noticeable changes in

length. In the rabbit intestinal ligated loop assay, activated LT causes the intestinal section treated with that toxin to produce noticeable accumulations of fluid (greater than 1ml/cm). The results of the tests (plate I-2 and I-3) show that the crude toxin preparation of *E.coli* JM_{83} ($pCHP_{21}$) and the culture supernatant, like the *E.coli* H10407, are all able to cause cells to produce positive morphological changes, and at the same time to produce a fluid accumulation in intestinal segments treated with toxin preparations. The *E.coli* JM_{83} (PUC_8) culture supernatant used as a control was not able to induce the above changes. Further experimentation showed that the above toxic effect can be neutralized by LT antiserum specificity or destroyed by boiling at 100°C for 10 minutes. The explanation is surely that the morphological changes of the cells is induced by heat-labile enterotoxin.

[illegible] 1 PIH [illegible] LT [illegible]
Table 1 The LT titers of strains in PIH test

[illegible] Strains	[illegible] (Titer)	
	[illegible] Crude toxin preparation	[illegible] Culture supernatant
E.coli JM_{83} ($pCHP_{21}$)	1:128	1:32
E.coli H_{10407}	1:8	ND*
E.coli 2050 (PJY_{11})	1:8	ND
E.coli JM_{83} (pUC_8)	ND	ND

ND*: Not detectable ([illegible])

We used external LWA recombination technology to isolate the LT gene from *E.coli* H10407 LT code plasmid pJY_{11}, and inserted it into the unitary PstI position of the pUC_8 carrier. The LT produced by the recombinant product had comparable immunity and biological activity as that produced by the parent strain H_{10407}; further, its level of expression was 16 times that of the parent strain's. Because the LT gene fragments in recombinant plasmid carry a activator (IPTG exhibits a non-induction effect with regard to the cloned strain LT), we deduce that the elevation of the LT expression level is a result of the increase in the recombinant plasmid copy number. That constructed by our laboratory expresses the swine dysentery epidemic vaccine strains of two antigens, K88ac and LT-B. Large scale experimentation shows that they have a rather good protective effect [12]. For this reason, the recovery of human LT gene clones will necessarily provide an effective tool for immunization protection against ETEC and for epidemiological studies. We have constructed the recombinant plasmid $pCHP_{21}$ of LTA-B (with 2.0 kb LT-B

gene fragments, see plate I-1), and obtained a level of expression similar to *E.coli* JM_{83} ($pCHP_{21}$). At the same time, because of the increase in the level of expression, the amount of LT-B antigen secreted to the exterior of the cell is also increased correspondingly; for this reason, it is to be hoped that it can be used in the construction of effective kinds of toxin vaccine strains.

REFERENCES

1. Yamamoto, T. et al. J. Bacteriol., 143:652, 1980.
2. Svennerholm, A.M. et al. Acta. Path. Microbiol. Immunol. Scand. Sect. C, 90:1, 1982.
3. Yamamoto, T. et al. J. Bacteriol., 152:506, 1982.
4. Yamamoto, T. et al. J Bacteriol., 145:850, 1981.
5. Li Fengsheng et al. Shengwu Gongcheng Xuebao, 3:102, 1987.
6. Birnboim, H.C. et al. Nucleic Acid Res., 7:1513, 1970.
7. Southern, E. et al. J. Mol. Biol., 98:503, 1975.
8. Li Shuqin et al. Junshi Yixuekexueyuan Yuankan, 10:255, 1986.
9. Evans, D.G. et al. Infect. Immun., 7:873, 1973.
10. Guerrant, R.L. et al. Infect. Immun., 10:320, 1974.
11. Chen Jinguang et al. Weishengwu Xuebao, 25:119, 1985.
12. Chen Tianmi et al. Zhongguo Shouyi Zazhi, 14:2, 1988.

EXPLANATION OF PLATE

1. Results of Southern hybridization and restriction analysis of recombinant plasmid pCHP$_{21}$.

   A. Ethidium bromide stained gel.
      a. PstI-digested pCHP$_{21}$ DNA.
      b. PstI-digested pJY$_{11}$ DNA.

   B. Results of Southern hybridization of the same gel.

   C. Result of restriction analysis of recombinant plasmid pCHP$_{21}$.
      a. pCHP$_{21}$ +PstI
      b. pCHP$_{21}$ +XbaI
      c. PCHP$_{21}$ +EcoRI + PstI
      d. pUC$_8$ + PstI
      e. PCHP$_{22}$ + EcoRI + PstI
      f. pCHP$_{21}$ + PstI + XbaI
      g. Lambda DNA + EcoRI - HindIII

2. Results of Chinese hamster ovary cell essay.

   A. CHO cells treated with *E.coli* JM$_{83}$(pUC$_8$) culture supernatant.
   B. CHO cells treated with *E.coli* JM$_{83}$(pCHP$_{21}$) culture supernatant.
   C. CHO cells treated with LT-antitoxin neutralized *E.coli* JM$_{83}$(pCHP$_{21}$) culture supernatant.

3. Results of adult rabbit intestinal ligated loop assay.

   A & E. Intestinal segments injected with *E.coli* JM83(pCHP$_{21}$) culture supernatant.
   B. Intestinal segment injected with *E.coli* JM$_{83}$(pUC$_8$) culture supernatant.
   C. Intestinal segment injected with *E.coli* [illegible]SO(pJY$_{11}$) culture supernatant.
   D & F. Intestinal segment injected with *E.coli* JM83(pCHP$_{22}$) culture supernatant.

DISTRIBUTION LIST

DISTRIBUTION DIRECT TO RECIPIENT

ORGANIZATION	MICROFICHE
C509 BALLISTIC RES LAB	1
C510 R&T LABS/AVEADCOM	1
C513 ARRADCOM	1
C535 AVRADCOM/TSARCOM	1
C539 TRASANA	1
C591 FSTC	4
C619 MIA REDSTONE	1
D008 MISC	1
E053 HQ USAF/INET	1
E404 AEDC/DOF	1
E408 AFWL	1
E410 AD/IND	1
F429 SD/IND	1
P005 DOE/ISA/DDI	1
P050 CIA/OCR/ADD/SD	2
AFIT/LDE	1
NOIC/OIC-9	1
CCV	1
MIA/PHS	1
LLYL/CODE L-309	1
NASA/NST-44	1
NSA/T513/TDL	2
ASD/FTD/TQIA	1
FSL	1